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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Pang et al.	Examiner: A. Kubelik
Serial No.	:	09/025,635	
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For	:	DNA CONSTRUCT TO CONFER MULTIPLE TRAITS ON PLANTS	

DECLARATION OF DENNIS GONSALVES UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, DENNIS GONSALVES, pursuant to 37 C.F.R. § 1.132, declare that:

1. I received a B.S. in Horticulture from the University of Hawaii in 1965; an M.S. in Plant Pathology from the University of Hawaii in 1968; and a Ph.D. in Plant Pathology from the University of California at Davis in 1972.

2. I was the Liberty Hyde Bailey Professor in the Department of Plant Pathology at Cornell University, Geneva, New York, from 1995-2002.

3. I am currently the Director of the Pacific Basin Agricultural Research Center of the USDA, Hilo, HI.

4. I am a co-inventor of the above-identified patent application.

5. This declaration is submitted to demonstrate that the disclosure of the present application would have enabled a skilled scientist to prepare additional DNA constructs having a fragment of a trait DNA from a viral source that has at least 110 nucleotides but is less than a full-length DNA, and a silencer DNA molecule, and to use such constructs to confer a desired trait (e.g., resistance against viral pathogens) to plants by transforming plants with such constructs.

6. Data presented in my patent application shows that multiple virus resistance can be obtained by transforming plants with a DNA construct that has a silencer DNA (e.g., 726 bp DNA of the green fluorescent protein (GFP) gene, an approximately 400 bp fragment of the nucleoprotein (NP) DNA of tomato spotted wilt virus (TSWV), or the turnip mosaic virus (TurMV) coat protein (CP) DNA) that is linked to short segments (about 200 bp to 87 bp) of tospovirus (groundnut ringspot virus, impatiens necrotic spot virus, and TSWV) coat protein nucleotide sequence (Example 7). This data also shows that when short DNA segments (200 bp or less) were used in a DNA construct without being linked to a silencer DNA molecule, the construct did not impart resistance to a plant transformed with that construct (Example 5). My patent application shows that resistance to different viruses can be achieved using my constructs, as demonstrated by the resistance imparted to transgenic plants against three tospoviruses, and against a potyvirus and tospovirus (Example 7, with Tables 5-6).

7. My previous work on developing transgenic papaya for resistance to papaya ringspot virus (PRSV) showed that some strains of PRSV could overcome the resistance in some transgenic papaya lines (Tennant et al., "Papaya Ringspot Virus Resistance of Transgenic Rainbow and SunUp is Affected by Gene Dosage, Plant Development, and Coat Protein Homology," European J. Plant Pathology 107(6):645-653 (2001) (attached hereto as Exhibit 1)). In particular, the transgenic 'Rainbow' papaya is commercially grown in Hawaii and virtually saved the papaya industry from destruction by PRSV. However, inoculation experiments have shown that 'Rainbow' is resistant to PRSV strains in Hawaii but is susceptible to a number of PRSV strains from outside of Hawaii. This differential resistance is largely due to the differences in nucleotide homology between the coat protein genes of different PRSV strains. In Hawaii, the PRSV strains share at least 97% homology to the PRSV coat protein transgene of 'Rainbow'. However, some strains, for example, YK from Taiwan and TH from Thailand, share only 89-90% homology to the CP transgene of 'Rainbow'. Thus, strains of PRSV from Taiwan or Thailand could cause severe damage to the Hawaiian papaya industry if they were introduced to Hawaii.

8. Recently, it was also reported from Okinawa that another potyvirus, papaya leaf distortion mosaic virus (PLDMV), causes symptoms similar to PRSV on papaya but is not related to PRSV (Maoka et al., "Nucleotide Sequence of

the Capsid Protein Gene of Papaya Leaf- Distortion Mosaic Potyvirus," Archives of Virology 141(1):197-204 (1996) (attached hereto as Exhibit 2). In fact, the coat protein of PLDMV shares only 49-59% amino acid similarity to the coat protein of PRSV. Greenhouse inoculations also showed that the 'Rainbow' papaya is susceptible to PLDMV. As with various PRSV strains, PLDMV could cause severe damage to the Hawaiian papaya industry if it was introduced to Hawaii.

9. Based on the success described above in paragraph 6, I believed the approach for obtaining resistance to multiple viruses or to strains of a virus as disclosed in my patent application could be used to develop Hawaiian transgenic papaya that would be resistant to outside strains of PRSV and simultaneously to PLDMV. Therefore, DNA constructs were prepared having a trait DNA that was less than a full-length trait-encoding DNA but containing at least 110 nucleotides, coupled to a DNA silencer molecule, with both the trait and the DNA silencer molecules under the control of a single 35S promoter and single terminator sequence. Various coding sequences for segments of the PRSV or PLDMV coat protein were incorporated into the DNA constructs. The constructs were then cloned into a suitable plant expression vector. Papaya plants were transformed with the expression vectors containing the DNA constructs, and the transgenic papaya were analyzed for resistance to the target viruses. The data from this work, described in detail herein *infra*, shows the effectiveness of the DNA constructs of my application in conferring multiple viral resistance to plants transformed with such constructs.

10. Using the techniques described in Example 1 of my patent application, basic gene constructs containing the green fluorescent protein (GFP) or one-half of the DNA molecule encoding the nucleocapsid protein (1/2NP) of TSWV under the control of a single promoter and terminator sequence, (i.e., [Promoter]-[GFP]-[DNA segments]-[Terminator] and [Promoter]-[1/2NP] [DNA segments]-[Terminator], respectively) were prepared. Various trait DNA fragments, described below, from a variety of PRSV strains, and/or PLDMV DNA segments (see 'Key to Figures 1A-N,' attached hereto as Exhibit 3, for a description of the components of the various DNA constructs), in translatable or non-translatable configurations, and a DNA useful for antibiotic transgene selection following transformation were inserted into these DNA constructs. Several of the transformation constructs prepared are shown in outline form in Figures 1A-1N (attached hereto as Exhibit 4).

11. Immature zygotic embryos extracted from seeds of immature green 'Sunrise' or 'Kapoho' papaya were transformed with expression vectors containing the DNA constructs prepared as described above and shown in Figures 1A-N (Exhibit 4). The transformation procedure was followed as described in Cai et al., "A Protocol for Efficient Transformation and Regeneration of *Carica papaya L. In Vitro*," Cell Devel. Biol-Plant 35: 61-69 (1999) (attached hereto as Exhibit 5). Transgenic embryos were regenerated in a medium containing the appropriate antibiotic for selection. Mature somatic embryos surviving selection were transferred to germination medium and allowed to develop into plantlets with dark green leaves and root initials. Plants were placed in rooting medium and transferred to the greenhouse. Transgenic lines from the germination medium were analyzed by PCR to confirm that the viral DNA was in the plantlets. Northern blots were carried out to detect the level of RNA expressed in transgenic lines, and the copy number of the transgene in the transgenic plants was determined by Southern blot analysis. Transgenic plants were further cultivated in accordance with conventional procedures so that the DNA construct was present in the resulting plants. To test viral resistance, plant inoculations with virus were done as described previously (Pang, S.-Z., et al., "Resistance to Heterologous Isolates of Tomato Spotted Wilt Virus in Transgenic Plants Expressing its Nucleocapsid Protein Gene," Phytopathology, 82: 1223-29 (1992)), and systemic symptoms of infection were recorded.

12. The results are summarized in Table 1 and Table 2 (attached hereto as Exhibits 6 and 7, respectively). The data from Table 1 show resistance against the PRSV strain from Keaau, Hawaii (KE), which is the first virus that the RO plants were tested against. The data clearly show that a segment of KE DNA (~ 200 nt in length) imparts resistance when linked to the silencer DNA. This demonstrates my patent application teaches constructs and a method for conferring resistance to additional viral plant pathogens, such as PRSV.

13. Plants that were resistant to KE were then screened for resistance to the other target viruses for which segments of the CP were present in the transgenic papaya (Table 2) (Exhibit 7). In other words, plants were first screened for resistance to KE and, subsequently, the KE-resistant plants were screened for resistance to the other strains of PRSV or to PLDMV. The data in Table 2 show that the approach of my present application provides resistance to various strains of PRSV and to PLDMV. In particular, two lines (numbers 493 and 494) with the DNA

construct pNP-YKT3'PLDMV showed resistance to PRSV strains of KE, TH, and YK, and to PLDMV. In addition, a transgenic papaya (line number 423) with DNA construct pNP-YKT5'Jap showed resistance to PRSV strains KE, YK, TH, and Jap. Thus, the approach described in my present patent application is enabling for conferring multiple viral resistance, e.g., for PRSV and for PLDMV.

14. The nucleotide sequences of many viral plant pathogens are known and available to the skilled scientist. For example, a single search request designating nucleotides for "viral plant protein" on the National Center for Biotechnology Information (NCBI) on-line search site generated a list of 288 nucleotide sequences for coat proteins of virus associated with plant pathogenesis that are available on the public database (see page 1 of NCBI search result attached hereto as Exhibit 8). It is common for research scientists to access publicly available viral genomic sequence information, carry out a BLAST or other type of homology search on that nucleotide relative to a second sequence of interest, and identify a potentially useful nucleotide sequence from a desired source for a given objective. Thus, it would be well within the capabilities of a skilled scientist to isolate or synthesize a fragment of 110 nucleotides or more of a desired trait DNA from a viral source for use in a DNA construct.

15. There are a multitude of protocols known to the skilled scientist that teach how to identify and isolate viral DNA, prepare DNA constructs in plant expression vectors, and to transform plants with such expression vectors. For example, Maoka et al. (Exhibit 2) and Allison et al., "Regeneration of a Functional RNA Virus Genome by Recombination Between Deletion Mutants and Requirements for Cowpea Chlorotic Mottle Virus 3a and Coat Genes for Systemic Infection," Proc Natl Acad Sci USA 87:1820-1824 (1990) (attached hereto as Exhibit 9) disclose methods to obtain and manipulate nucleic acid molecules from plant viruses. Many other useful references are available which provide specific guidance to the skilled scientist for the transformation, regeneration, and testing of transgenic plants. For example, Gene Transfer to Plants, Potrykus and Spangenberg, eds., Springer Verlag Press, Berlin (1995) (see generally, the List of Contents for Gene Transfer to Plants, attached hereto as Exhibit 10), discloses methods for gene transfer to plants using *Agrobacterium*-mediated gene transfer (Parts I and II), protoplast transfer (Part III), biolistic transformation (Part IV), microinjection and fiber-mediated transformation (Part V), and tissue electroporation (Part VI), as well as methods for the analysis of

transgenic plants post-transformation (Part VII), the establishment and maintenance of embryogenic cultures (Part VIII), and the use of genetic markers and expression signals in plant transformation (Part IX).

16. Therefore, it is clear that a skilled scientist having read my present patent application would know how to make additional DNA constructs having a fragment of a trait DNA from a viral source that has at least 110 nucleotides but is less than a full-length DNA, and a silencer DNA, under the control of a single promoter and terminator, and how to use such constructs to prepare expression vectors and host cells, including plant cells, containing those nucleic acid molecule constructs, and, finally, how to prepare transgenic plants to impart a desired trait to the transformed plants.

17. I hereby declare that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 29, 2003

Dennis Gonsalves, Ph.D.